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Functional Knockout of the Adenosine 5′-Phosphosulfate Reductase Gene in *Physcomitrella patens* Revives an Old Route of Sulfate Assimilation*

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The reduction of adenosine 5′-phosphosulfate (APS) to sulfite catalyzed by adenosine 5′-phosphosulfate reductase is considered to be the key step of sulfate assimilation in higher plants. However, analogous to enteric bacteria, an alternative pathway of sulfate reduction via phosphoadenosine 5′-phosphosulfate (PAPS) was proposed. To date, the presence of the corresponding enzyme, PAPS reductase, could be neither confirmed nor excluded in plants. To find possible alternative routes of sulfate assimilation we disrupted the adenosine 5′-phosphosulfate reductase single copy gene in *Physcomitrella patens* by homologous recombination. This resulted in complete loss of the correct transcript and enzymatic activity. Surprisingly, the knockout plants grew on sulfate as the sole sulfur source, and the concentration of thiols in the knockouts did not differ from the wild type plants. However, when exposed to a sublethal concentration of cadmium, the knockouts were more sensitive than wild type plants. When fed [^{35}S]sulfate, the knockouts incorporated ^{35}S in thiols; the flux through sulfate reduction was ~50% lower than in the wild type plants. PAPS reductase activity could not be measured with thioredoxin as reductant, but a cDNA and a gene coding for this enzyme were detected in *P. patens*. The moss *Physcomitrella patens* is thus the first plant species wherein PAPS reductase was confirmed on the molecular level and also the first organism wherein both APS- and PAPS-dependent sulfate assimilation co-exist.

Assimilatory sulfate reduction is a pathway used by plants, algae, fungi, and bacteria to convert inorganic sulfate to sulfide, which is further incorporated into carbon skeletons of amino acids to form cysteine or homocysteine (1). For reduction, sulfate must be activated by ATP-sulfurylase to adenosine 5′-phosphosulfate (APS).[1] In higher plants, APS is directly reduced by APS reductase (APR) to sulfite, which is subsequently reduced to sulfide by sulfite reductase and, as such, is incorporated into *O*-acetylserine by *O*-acetylserine (thiol)lyase (Fig. 1) (2, 3). In fungi and some enteric bacteria, such as *Escherichia coli* and *Salmonella typhimurium*, APS must be phosphorylated by APS kinase to PAPS in order to be reduced by PAPS reductase (4). Because the sulfate assimilation pathway was first resolved in bacteria that reduce sulfate via PAPS, the PAPS reductase pathway of sulfate assimilation was expected to exist also in plants (5, 6). Although plants and algae utilize APS rather than PAPS as a sulfonyl donor for reduction, and APR was shown to play a key role in controlling sulfate reduction in plants (2), the existence of a PAPS-dependent pathway of sulfate assimilation was never excluded, especially because a partial purification of a PAPS reductase from spinach had been reported (7). The *Arabidopsis* genome does not contain any genes homologous to *E. coli* PAPS reductase other than those encoding three isoforms of APR (8, 9). Because a putative plant PAPS reductase may have a structure completely divergent from that of the bacterial enzyme, only an analysis of plants lacking APR activity may prove or exclude the PAPS-dependent sulfate assimilation in plants. However, until now neither mutants nor transgenic plants devoid of APR activity were described, probably because APR is encoded by small multigene families of two or three isoforms in most plant species analyzed to date (8–11). Therefore, to produce and analyze plants lacking APR activity we turned to the moss *Physcomitrella patens*.

In the last few years, *P. patens* became a model system used increasingly to study the function of plant genes (12–14). *Physcomitrella* can easily be stably transformed by polyethylene glycol-mediated DNA transfer to protoplasts (15). Most importantly, it possess an efficient system of homologous recombination allowing exact gene targeting (16–19). Thus, *Physcomitrella* is the only plant wherein gene knockouts can be routinely produced by a highly efficient and straightforward approach (20). Using this approach, a function of an ancestral tubulin FtsZ in plastid division was revealed (17). The value of the targeted gene disruptions was further demonstrated by an analysis of knockouts in genes encoding Δ6-acyl group desaturase and the multiubiquitin chain binding subunit MCB1 of the 26 S proteasome, illustrating their functions in fatty acid synthesis and plant development, respectively (18, 19). Here, we report an analysis of a functional knockout in a gene encoding

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ429099 and AJ489220.

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[1] The abbreviations used are: APS, adenosine 5′-phosphosulfate; APR, APS reductase; PAPS, phosphoadenosine 5′-phosphosulfate; EST, expressed sequence tag; HPLC, high pressure liquid chromatography; CHES, 2-(cyclohexylamino)ethanesulfonic acid; contig, group of overlapping clones.

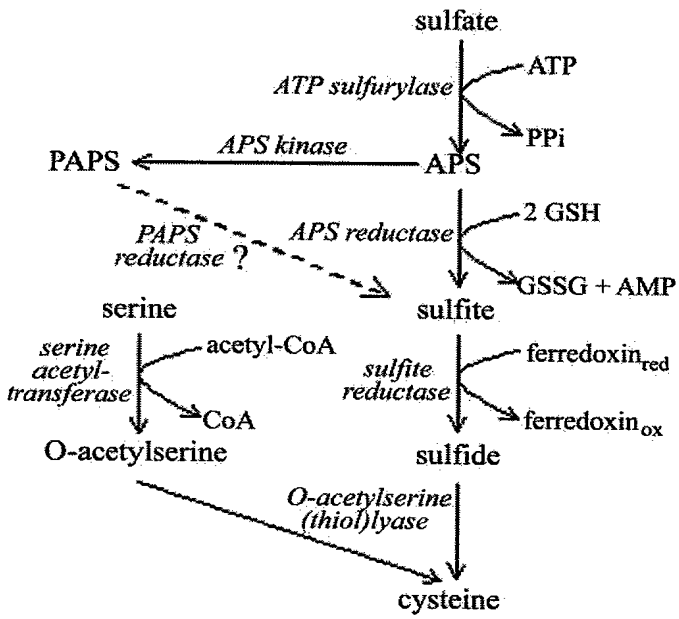

FIG. 1. **Pathway of assimilatory sulfate reduction in plants.**

APR in *P. patens* and the impact of this knockout on sulfate assimilation.

EXPERIMENTAL PROCEDURES

Plant Material—Physcomitrella patens (Hedw.) B.S.G. was cultured in liquid or solid modified Knop's medium as described earlier (21). For protoplast isolation, protonema was grown in semi-continuous bioreactor cultures supplemented with 2.5 mM ammonium tartrate (22). To study the effect of cadmium, the plants were grown for 5 days on solid Knop's media supplemented with 5, 10, or 15 μM $CdCl_2$.

Protoplast Isolation, Transformation, and Regeneration—Protoplasts were isolated and transformed as described previously (17). The transformations were performed with 3×10^6 protoplasts and 30 μg of DNA. Regeneration and selection were performed as described (17). For selection, Knop's media were supplemented with 50 μg/ml G418. Both selection and release media contained 5 μg/ml reduced GSH.

Molecular Cloning—The cDNA clone for APR (*PpAPR*, pp004074261r) was obtained upon a BLAST search of the *P. patens* EST library, which was generated in a joint project of the University of Freiburg and BASF Plant Science GmbH,[2] with the protein sequence of the APR2 isoform of APR from *Arabidopsis thaliana* (GenBank™ accession no. U56921) as a query. The 1724-bp full-length *PpAPR* cDNA clone was sequenced on both strands. The corresponding genomic DNA (3616 bp) was obtained by PCR and sequenced by primer walking. To create the knockout construct, a fragment of 2006 bp was amplified from genomic DNA by PCR with primers R10 (TCTTTCACTATTCGGTGACG) and R11 (CGACCACAACATTAGATCC) (Fig. 2) and cloned into pCRII vector (Invitrogen). This plasmid was digested with *Hin*dII to cut out a 361-bp fragment containing part of the fifth and the sixth exon (Fig. 2) and replace it with the *nptII* selection cartridge, which was obtained from the vector pRT101neo (18). To clone the selection cassette, pRT101neo was digested with *Hin*dIII, and the protruding ends were filled with the Klenow fragment of DNA polymerase (24). For transformation, 30 μg of the knockout construct were cut with *Eco*RI, producing a 3-kb linear fragment that contained the *nptII* gene flanked by APR genomic sequences of 874- and 771-bp in length. The DNA was precipitated from the reaction mixture, redissolved in 90 μl of water, and used for transformation of *P. patens*. *P. patens* PAPS reductase sequences (GenBank™ accession nos. BJ166495 and BJ173474) were retrieved from the GenBank™ after a TBLASTN search of the EST data base with the PpAPR protein sequence as a query. Oligonucleotide primers PR1 (CTTACTTTGTACAATTAGAAGG) and PR2 (TAAGTTTCTCAGCGAAGTGG) were derived from these EST sequences and utilized to amplify the cDNA from *P. patens* total RNA by reverse transcription PCR. The identity of the PCR fragment was confirmed by sequencing.

Molecular Analysis of Transgenic Plants—For the prescreening of G418-resistant plants, small pieces of gametophores (1–5 mg) were treated for 30 min at 45 °C in 75 mM Tris-HCl, pH 8.8, containing 20 mM $(NH_4)_2SO_4$ and 0.1% Tween 20, and 3 μl of this extract was used for PCR with the following four pairs of primers (Fig. 2): R14 (CGGAACGCGTGCCAACGTTC) and R15 (GCAACACCATCAAGGTTACC) to detect a disruption of the original *apr* gene, N1 (TACCGACAGTGGTCCCAAAG) and N2 (CCACCATGATATTCGGCAAG) to detect the presence of the *nptII* cassette, R20 (CAAAGCACCGTTGATATCC) and N3 (TGTCGTGCTCCACCATGTT) to control the integration of the transgene at the 5′ end, and N4 (GTTGAGCATATAAGAAAC) and R21 (TGCGTAGGCTTTCTGAGC) to check the integration at the 3′-end. Plants that gave the expected fragments by all four PCR reactions were considered as putative knockouts and selected for further analysis. For Southern analysis, genomic DNA was isolated from the selected plants with the Nucleospin plant kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions, and the analysis was performed with 2 μg of DNA according to standard protocol (24). The integration regions of the transgene were isolated from transformants 11-3-3, 11-3-11, 11-3-12, 12-4-29, and 12-7-7 by PCR with primer pairs R20 and N3 and R21 and N4 for the 5′- and 3′-ends, respectively. The PCR fragments were cloned in pCRII vector and sequenced.

Total RNA was isolated from moss tissue using the RNeasy kit (Qiagen, Hilden, Germany). Reverse transcription PCR with primers R14 and R11 was performed according to the standard protocol (24). As control, the reverse transcribed RNA was subjected to PCR with primers C45F (GGTTGGTCATGGGTTGCG) and C45R (GAGGTCAACTGTCTCGCC) corresponding to the constitutively expressed gene for the ribosomal protein L21 (25). For Northern analysis, 5 μg of total RNA were subjected to electrophoresis on formaldehyde-agarose gels at 120 V, transferred onto Hybond-N nylon membranes (Amersham Biosciences), and hybridized with ^{32}P-labeled cDNA probes for APR, L21, and PAPS reductase. The membranes were washed four times at different concentrations of SSC in 0.1% SDS for 20 min at 65 °C (the final washing step being 0.5× SSC, 0.1% SDS at 65 °C) and exposed to x-ray film (Fuji medical RX) at −80 °C for 2 to 3 days.

Enzyme Assays—Plant extracts were prepared from 200 mg of protonema tissue by homogenization in 2 ml of 50 mM Na/KPO_4 buffer (pH 8) supplemented with 30 mM Na_2SO_3, 0.5 mM 5′-AMP, and 10 mM dithioerythritol (26). APR activity was measured in extracts as the production of [^{35}S]sulfite, assayed as acid-volatile radioactivity, formed in the presence of [^{35}S]APS and dithioerythritol (27). PAPS reductase was measured using [^{35}S]PAPS, dithioerythritol, and recombinant thioredoxin m or f from spinach, thioredoxin from *E. coli*, or GSH and glutaredoxin from *E. coli* (28). Protein concentration in the extracts was determined according to Bradford (29) with bovine serum albumin as standard.

HPLC Analysis of Low Molecular Weight Thiols—For *in vitro* labeling, 50–100 mg of plant material from liquid culture was ground in liquid nitrogen and extracted in 750 μl of 0.1 N HCl. After centrifugation, 120 μl of supernatant were mixed with 180 μl of 0.2 M CHES buffer, pH 9.3, and 30 μl of 5 mM dithiothreitol were added to reduce disulfides. After reduction, 20 μl of 30 mM monobromobimane (Thiolyte® MB, Calbiochem) was added, and derivatization of thiols was allowed to proceed for 15 min in the dark. The reaction was stopped, and the conjugates were stabilized by adding 240 μl of 10% acetic acid. Bimane conjugates were separated by HPLC (SUPELCOSIL™ LC-18, 25 cm × 4.6 mm, 5 μm, Sigma-Aldrich) using 10% (v/v) methanol, 0.25% (v/v) acetic acid (pH 3.9) as solvent A and 90% (v/v) methanol, 0.25% (v/v) acetic acid (pH 3.9) as solvent B. The elution protocol employed a linear gradient from 96 to 82% A in 20 min, and the flow rate was kept constant at 1 ml/min. Bimane derivatives were detected fluorimetrically (RF535, Shimadzu, Kyoto, Japan) with excitation at 390 nm and emission at 480 nm.

Feeding of $^{35}SO_4^{2-}$ and Determination of ^{35}S in Thiols—Three cultures each of wild type moss and the 11-3-12 knockout line containing ~100 mg of protonema in 10 ml of Knop's medium with 0.06 mM SO_4^{2-} were supplemented with 150 μCi of $^{35}SO_4^{2-}$ (specific activity 9.25×10^{12} Bq mmol^{-1}) and further cultivated for 2 h. Plant material was extracted with 1.2 ml of 0.1 M HCl containing 1 mM Na_2EDTA. After centrifugation, the thiols were reduced with dithiothreitol and labeled with monobromobimane as described (30). A 100-μl aliquot of each sample was separated by HPLC, and fractions of 1 ml were collected in scintillation vials. The ^{35}S radioactivity was determined in a liquid scintillation

[2] S. Rensing, S. Rombauts, A. Hohe, D. Lang, E. Duwenig, P. Rouze, Y. Van de Peer, and R. Reski, submitted for publication. Manuscript available at www.plant-biotech.net/Rensing_et_al_transcriptome2002.pdf.

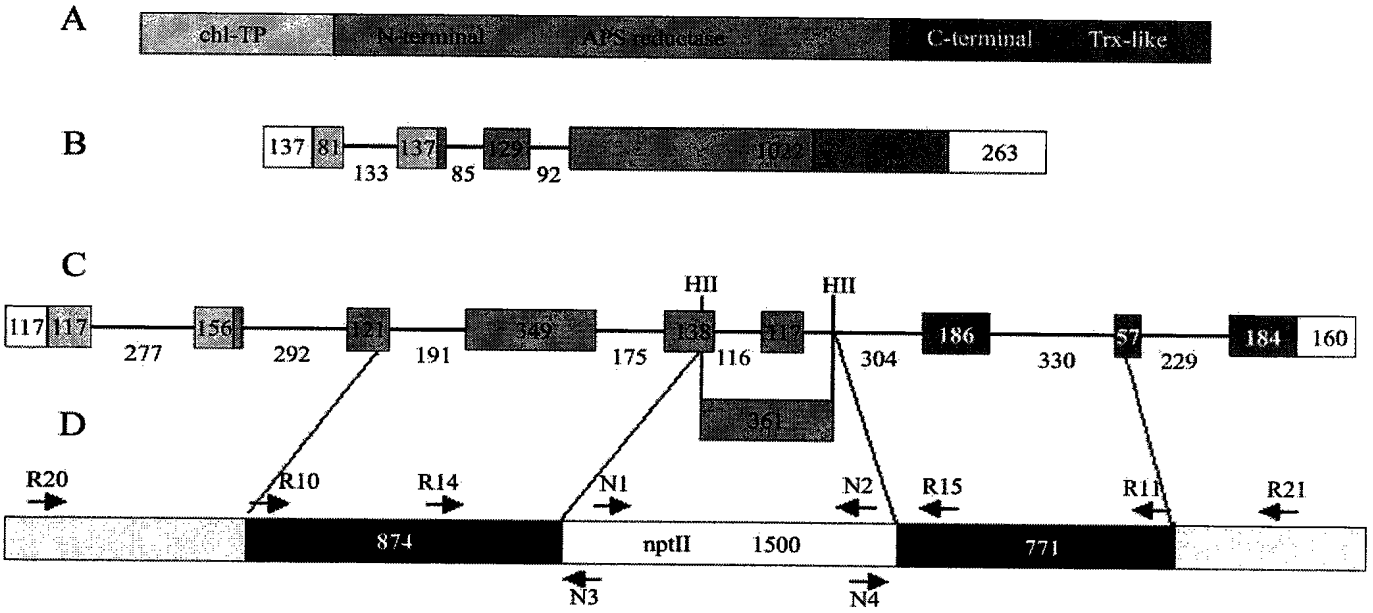

FIG. 2. **Schematic representation of the APR gene and knockout construct.** *A*, schematic representation of the three domains of APR protein (*chl-TP*, chloroplast targeting peptide; *Trx*, thioredoxin). *B*, the *apr2* gene from *A. thaliana*. The *rectangles* represent exons; the colors correspond to the respective domains; *white* represents 5′ and 3′ non-translated regions. Introns are presented as *lines*. The numbers represent lengths in base pairs. *C*, the *apr* gene from *P. patens*. The position of the two *Hin*dII sites, utilized to cut out the 361 fragment, are indicated. *D*, the *apr* disruption construct. The position of the PCR primers is indicated by the *arrows*.

counter (Wallac, Turku, Finland). Total cysteine, γ-glutamylcysteine, and GSH were analyzed by the same HPLC system as described above.

RESULTS

Cloning and Characterization of the APS Reductase Gene—The sequence of the APR2 isoform of APS reductase from *A. thaliana* was used as a query to search the *P. patens* EST bank, which was generated in a joint project of the University of Freiburg with BASF Plant Science GmbH,[2] with the BLAST software. The analysis identified a single contig composed from 16 sequences. The longest cDNA clone, pp004074261r, was fully sequenced. This cDNA was 1724 base pairs long and 57.9% identical to the APR2 cDNA. It contained a single open reading frame encoding a protein of 465 amino acids. Similar to APR from *Arabidopsis*, the protein from *P. patens* consisted of the following three domains: a chloroplast-targeting peptide, a part similar to bacterial PAPS reductase, and a C-terminal thioredoxin-like domain (Fig. 2). The APR of *P. patens* was 60.6% identical to the APR2 at the amino acid level; the identity was raised to 71.3% when the mature proteins were compared. Surprisingly, the thioredoxin-like active site C*XX*C was modified to C*XX*S. Southern analysis confirmed that, in contrast to higher plants, APR is encoded by a single gene in *P. patens* (data not shown). The APR cDNA sequence was used to define oligonucleotide primers for amplification of the gene from the genomic DNA. The gene was then completely sequenced. Fig. 2 shows the exon/intron structure of the APR-encoding gene from *P. patens* and its comparison with the *apr2* gene from *A. thaliana* (31). The three introns present in the *A. thaliana apr2* gene were found at identical positions in *P. patens*. However, whereas the major part of the protein is encoded by a single exon in *Arabidopsis*, five additional introns are present in the *P. patens* gene (Fig. 2).

Gene Targeting—To synthesize the disruption construct, a 2006-bp central part of the APR gene was amplified from the genomic DNA (Fig. 2), cloned into the pCR plasmid, and a 361-bp fragment comprising part of exon 5, intron 5, and exon 6 was replaced by the *nptII* gene as a selection marker. To identify positive recombination events, 130 regenerated G418-resistant plants were prescreened by PCR with four different primer combinations to detect the *nptII* cassette, the disruption of the *apr* gene, and the correct integration of the transgene on both the 5′- and 3′-ends. For 55 transformants, all four PCR reactions resulted in the expected products, indicating that these represent true knockouts of the *apr* gene. From these, eight individual transformants were selected for further analysis. Southern blot analysis using the 361-bp fragment (which had been replaced in the construct by the *nptII* gene) as a probe verified that, in all eight transformants, the original APR gene was disrupted (Fig. 3). Sequencing of the 5′- and 3′-integration sites of the transgene from five transformants revealed precise integrations without any insertions or deletions. Flow cytometry measurements revealed that the transformant 12-4-2 was a diploid, whereas the other seven mutants were haploids.

No APR transcript was detected in the transformants by reverse transcription PCR with primers R14 and R15 (Fig. 4*A*). Northern analysis detected the correct 1.6-kb APR transcript solely in the wild type, whereas the transformants possessed only aberrant transcripts of higher or lower molecular weight (Fig. 4*B*). The expression of the control gene for L21 (25) was not affected in the transformants.

Analysis of Sulfate Assimilation—To answer the question of whether the disruption of the *apr* gene indeed resulted in loss of function, APR activity was measured in the transformants. Although APR activity reached 6.1 nmol min^{-1} mg $protein^{-1}$ in extracts of wild type plants, sulfite production from APS could not be measured in the extracts of the transformants. The transgenic plants were thus real functional knockouts in APS reductase.

Because we expected that the transgenic plants lacking APR would not be able to grow on sulfate as a sole sulfur source like yeast or *E. coli* PAPS reductase mutants (32, 33), the selection media were supplemented with GSH. Surprisingly, however, when transferred to a normal Knop's medium without a reduced sulfur source, the transgenic plants survived and showed no obvious phenotypic effects compared with the wild type. The

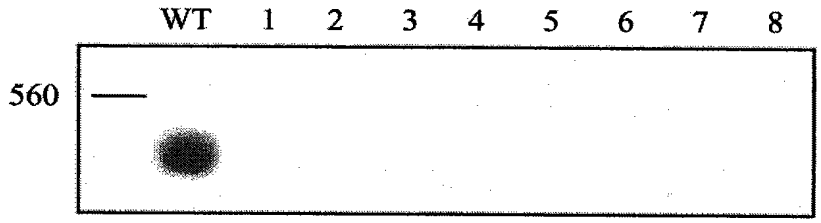

FIG. 3. **Southern blot analysis.** Genomic DNA was extracted from protonema tissue of wild type *P. patens* (*WT*) and eight Δapr knockouts: 11-3-3 (*lane 1*), 11-3-11 (*lane 2*), 11-3-12 (*lane 3*), 12-4-2 (*lane 4*), 12-4-3 (*lane 5*), 12-4-14 (*lane 6*), 12-4-29 (*lane 7*), and 12-7-7 (*lane 8*). The DNA was cleaved with *Hin*dII, separated on 1% agarose, blotted onto Hybond-N nylon membrane, and hybridized with a ^{32}P-labeled 361-bp fragment of *apr* gene, which was replaced in the disruption construct by the *nptII* cassette.

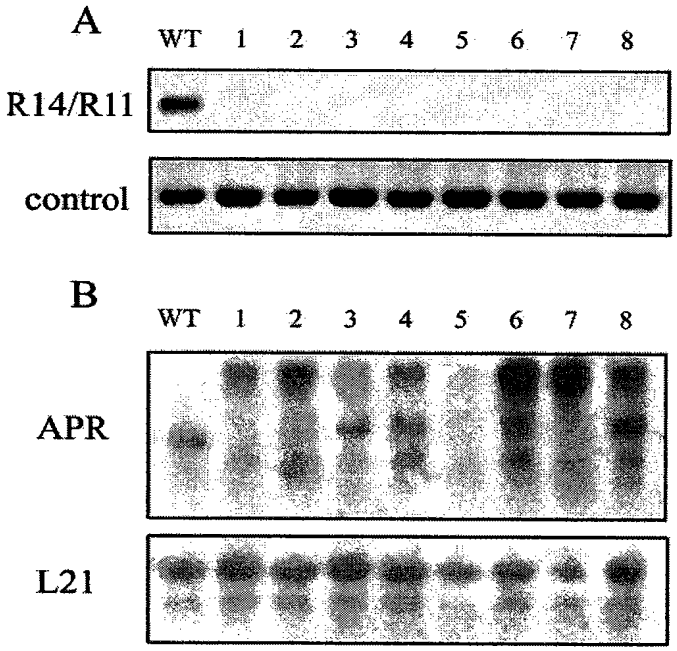

FIG. 4. **Expression analysis of APR.** Total RNA was extracted from protonema tissue of wild type *P. patens* (*WT*) and 8 Δapr knockouts: 11-3-3 (*lane 1*), 11-3-11 (*lane 2*), 11-3-12 (*lane 3*), 12-4-2 (*lane 4*), 12-4-3 (*lane 5*), 12-4-14 (*lane 6*), 12-4-29 (*lane 7*), and 12-7-7 (*lane 8*). *A*, 2 μg of RNA were reverse transcribed, and an equivalent of 200 ng was utilized for PCR with primers R14 and R11 to detect the APR transcript. The PCR fragments were resolved on 1% agarose/Tris borate EDTA gel. As control, PCR was performed with primers derived from the sequence of the gene for ribosomal protein L21. *B*, 5 μg of RNA were separated on 1% agarose in the presence of formaldehyde, blotted onto Hybond-N nylon membrane, and hybridized with ^{32}P-labeled cDNA fragments of APR and L21.

determination of low molecular weight thiols revealed no significant difference in the concentration of cysteine and glutathione between wild type and mutant plants (data not shown). Evidently, an alternative pathway of sulfate reduction independent of APR must exist in *P. patens*.

To test whether this alternative pathway is also capable of providing enough reduced sulfur under conditions of increased demand, the transformants were exposed to three different sublethal concentrations of cadmium. After 5 days of incubation on 15 μM $CdCl_2$, the wild type plants were seriously damaged but still viable, whereas the transformants were dead (Fig. 5). At lower cadmium concentrations, the transformants were also injured more than the wild type plants. To obtain quantitative data about the flux through the sulfate assimilation pathway, plants of the wild type and the 11-3-12 Δapr line were fed [^{35}S]sulfate, and the radioactivity incorporated into cysteine and GSH was determined (Fig. 6). The knockout was indeed able to reduce sulfate but at a significantly lower rate. The flux from sulfate toward thiols was 40% lower in the knockout line than in the wild type plants.

Identification of PAPS Reductase in Physcomitrella patens—To identify the enzyme(s) involved in the alternative sulfate assimilation pathway of the knockouts, we first measured the PAPS reductase activity. Neither the wild type moss nor the transformants possessed measurable activities of this enzyme using dithiothreitol and recombinant thioredoxin m and f from spinach, thioredoxin from *E. coli*, or glutaredoxin and GSH as reductants (data not shown). However, a new search of the GenBank™ data base with the *P. patens* APR as a query identified two recently submitted EST clones (GenBank™ accession nos. BJ166495 and BJ173474) as putative PAPS reductase. The deduced amino acid sequence is 24.2% and 31.1% identical with *P. patens* APR and *E. coli* PAPS reductase, respectively (Fig. 7). The putative protein contains the (P)APS reductase signature ECG(I/L)H but not the two cysteine pairs binding the FeS cluster in APR (34). The corresponding cDNA was amplified from *P. patens* RNA and used as a probe for Northern analysis. PAPS reductase was indeed expressed in wild type and all Δapr knockout lines analyzed. In most of the knockouts, the mRNA level was two to three times higher than in the wild type moss (Fig. 8). *P. patens* is thus the first plant species with molecular evidence for a PAPS reductase and also the first organism where both APS- and PAPS-dependent reductases exist.

DISCUSSION

APS reductase is an enzyme possessing major control over the flux through sulfate assimilation in plants and, as such, is extensively regulated (1, 2). No mutants or transgenic plants lacking the APR have been described; however, the deletion of its counterpart in bacteria and yeast, the PAPS reductase, led to cysteine auxotrophy of the mutants (32, 33). In most higher plants, APR is encoded by a small multigene family (8–11). This redundancy might explain why no APR-deficient mutants have been identified to date. Here we have shown that the moss *P. patens* contains only a single gene for APR. The *apr* gene structure from *P. patens* differs significantly from the *apr* genes of *A. thaliana*. The three introns present in the *A. thaliana apr2* and *apr3* genes (*apr1* is lacking intron 2) were found at identical positions in *P. patens*, but five additional introns are present in the *P. patens* gene. The mature APR protein is composed of two domains catalyzing separate steps in the reduction of APS (35). It was therefore expected that the protein originated from a fusion of the individual proteins and that these domains would be encoded by discrete exons (31). However, surprisingly, the major part of the protein is encoded by a single exon in *Arabidopsis* (31). In the *P. patens apr* gene, the two domains are separated by an intron (Fig. 2); thus, the *apr* gene organization in *P. patens* but not in *A. thaliana* corresponds to its probable evolutionary history, *i.e.* the fusion of genes for bacterial type APR and thioredoxin (36).

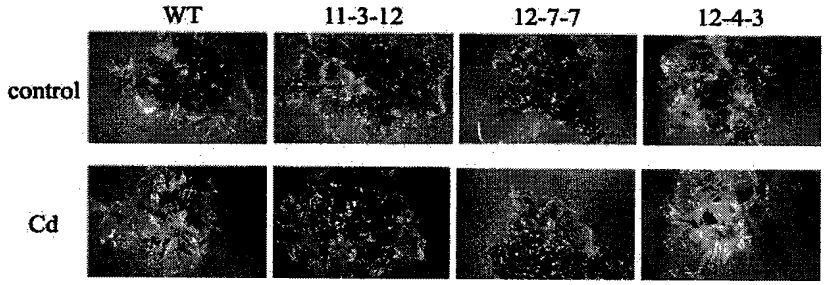

FIG. 5. **Cadmium sensitivity of Δapr knockouts.** Wild type *P. patens* and three Δapr knockouts were incubated for 5 days on solid Knop's medium (control) or Knop's medium with the addition of 15 μM $CdCl_2$ (Cd).

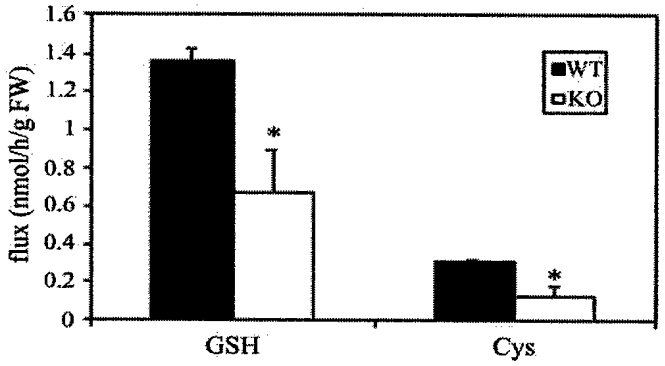

FIG. 6. **Incorporation of ^{35}S from [^{35}S]sulfate into thiols.** Wild type moss and the 11-3-12 Δapr knockout were incubated in Knop's medium containing 0.06 mM sulfate supplemented with $^{35}SO_4^{2-}$ for 2 h. Radioactive sulfur in cysteine and GSH was measured. Mean values ± S.D. from three measurements are presented. Values indicated by *asterisks* are different at $p \leq 0.005$.

P. patens seems to be an excellent species in which to construct plants lacking APR activity because of the possibility of gene targeting in this organism (13, 16, 20) and the presence of only a single *apr* gene in the genome. The *apr* gene was disrupted by the substitution of a 361-bp gene fragment by the *nptII* cassette via homologous recombination. In 55 of 130 G418 resistant plants analyzed, all four criteria for selecting putative knockouts were met. The efficiency of the gene targeting (42%) was thus higher than in experiments in which cDNA was used for the disruption constructs (17, 19) and comparable or lower than described for constructs with genomic DNA (18, 36). In all eight randomly selected putative knockouts, Southern analysis (Fig. 3) revealed the disruption of the wild type *apr* locus, and the sequencing of the junction regions of five transformants confirmed a correct integration of the transgene in the genome, similar as described previously (17, 18, 37). The disruption of the *apr* gene led to a loss of a correct transcript as revealed by reverse transcription PCR and Northern analysis (Fig. 4). The latter method detected a single APR transcript in the wild type *P. patens* and strong but aberrant signals of both greater and lower molecular weight in the knockouts (Fig. 4). The aberrant transcripts, which were also observed by Strepp *et al.* (17), were most probably derived from the still functioning native *apr* promoter or from the insertion of multiple copies of the transgene. The lack of a correct transcript thus corroborates the disruption of the *apr* gene. However, the absence of an APR transcript still was not sufficient evidence for a full lack of APR function, because the possibility of a second isoform with a different primary structure could not be excluded. Therefore, only the absence of detectable APR activity represented the final proof that the Δapr knockouts obtained indeed represent mutants with a complete lack of function.

The primary reason for construction of the APR knockouts was to bring evidence for or against the existence of PAPS reductase in plants. This enzyme was proposed to be present in plants analogous to enteric bacteria and yeast (5, 6) and its activity was measured in spinach (7); its existence is, however, rather doubtful (2). Indeed, in the *Arabidopsis* genome, no homologue of *E. coli* PAPS reductase other than the three APR isoforms was found. However, as discussed above, the lack of homologue proteins does not exclude an existence of a protein with a completely unrelated primary structure. Due to the presence of APR, the PAPS reductase activity could be masked because the two sulfonucleotides are easily interchangeable by catalytic action of APS kinase and 3′(2′),5′-bisphosphate nucleotidase or because the enzyme synthesis might be repressed. Therefore, by the disruption the *apr* gene we hoped to remove the possible repression of PAPS reductase. We expected that the Δapr knockouts would be disturbed in sulfate assimilation and therefore might be unable to grow on sulfate as the sole sulfur source like mutants of *E. coli* or *Saccharomyces cerevisiae* lacking functional enzymes of the sulfate assimilation pathway (32, 33). Surprisingly, however, the knockout plants grew also on a standard medium without the addition of reduced sulfur, and under normal growth conditions they were undistinguishable from wild type plants. Clearly, another route of sulfate reduction independent from APR must exist in *P. patens*. This new pathway seems to be as efficient as the APR-dependent sulfate assimilation, because the knockouts did not exhibit any phenotypical changes when grown on a standard nutrient solution with sulfate as the sole sulfur source. It was thus interesting to test the efficiency of the novel pathway under conditions of increased demand of reduced sulfur. In higher plants, exposure to cadmium leads to the induction of synthesis of phytochelatins, small peptides formed from repetitions of γ-glutamylcysteine units (38). In most bryophytes, phytochelatins are not synthesized, but the glutathione pool increases significantly upon the addition of cadmium (39). In any case, the exposure of plants to heavy metals leads to an increased demand for reduced sulfur compounds. The treatment of the Δapr knockouts with cadmium revealed strong phenotypical differences with the wild type plants under these conditions. Thus, the new pathway was not as effective in coping with the higher demand for glutathione as is the pathway via APR. The confirmation that the cadmium-sensitive phenotype of the Δapr knockouts indeed is caused by a reduced capacity for sulfate reduction was achieved by *in vivo* measuring of flux through sulfate assimilation (Fig. 6).

The most obvious explanation for the alternative route of sulfate reduction would be the presence of PAPS reductase. In the Δapr knockouts, however, no PAPS reductase activity could be measured. Nevertheless, an EST sequence from *P. patens* was identified that most probably codes for PAPS reductase (Fig. 7) because of the following. (i) The deduced amino acid sequence is 31.1% identical to PAPS reductases from *E. coli* and yeast. (ii) The sequence contains the (P)APS reductase signature ECG(IL)H (36) but not the two cysteine pairs binding the FeS cluster in APR (34, 36). (iii) The mRNA level of this EST was increased in most of the Δapr knockouts (Fig. 8).

```
AtAPR2   MALAVTSSSTAISGSSFSRSGASSESKALQICS---------IRLSDRTHLAQRRPMKPLNAESHSRSESWVTRASTLIA   71
PpAPR    MALKVASLEAAVMASLAPAASTSASLSRQSIVSGLAFRGQNGFQLGGLSSAGVSSRSLALGGKKIELDIAQPRRMLPVSA   80
PpPAPR   MAMAMADAHLRSSTSCVPSHCPALVPALATLTS------LRKCAFHKPSFPSLVFQSYCLGWASGRGKTAASAVPVTTSG   74
EcPAPR                                                          MSKLDLNALN-ELPKVDRILALAE   23
                                                                   *

AtAPR2   PEVE-EKGGEVEDFEQLAKKLEDASPLEIMDKALERFGDQIAIAFSGAEDVALIEYARLTG----KPFRVFSLDTGRLNP  146
PpAPR    TAVVSESVQSTVDIPKLAEKLDSASPLEIMDKALSLFGDDIAIAFSGAEDVTLIEYAHLTG----RPFRVFSLETGRLNP  156
PpPAPR   SSIDIKKCNEQARDARLQHLEAQA--LETLQKTVENFEKPAFPCALIAGDVVILDLLHRIGAFSDNKVKIIFIDTFHLFP  152
EcPAPR   TNAELEKLDAEGRVAWALDNLP---------------GEYVLSSSFGIQAAVSLHLVNQIR----PDIPVILTDTGYLFP   84
                                                                           *  * *

                                                ⇓⇓
AtAPR2   ETYRLFDAVEKQYGIRIEYMFPDAVEVQALVRNKGLFSFYEDGHQECCRVRKVRPLRRALKGL--KAWITGQRKDQSPGT  224
PpAPR    ETLKLFDDVEKRYNIRIQYMYPDAAEVEELTRTKGLFSFYEDGHQECCRVRKVRPLRRALKGL--KAWITGQRKDQSPGT  234
PpPAPR   ETYKFLSEVEERYGFKAHVFHAADVNNKEAYDAKFGSDLFITDIEEYDRICKVEPFSRALKTLEVDAMINGRRRDHG-AE  231
EcPAPR   ETYRFIDELTDKLKLNLKVYRATESAAWQEARYGKLWEQGVEGIEKYNDINKVEPMNRALKELNAQTWFAGLRREQS-GS  163
         **                                                 ** *  **** *       * *
                                                            ⇓  ⇓
AtAPR2   RSEIPIVQVDPVFEGLDGGVGSLVKWNPLANVEGADVWNFLRTMDVPVNALHAQGYVSIGCEPCTRPVLPG-----QHER  299
PpAPR    RANVPVVQVDPAFEGLDGGPGSLVKWNPLSNVSGTAVWSFLRTMDVPVNALHFKGYVSIGCEPCTRAVLPG-----QHER  309
PpPAPR   RAHLEVFEE-----------GKMVKVQPLAYWEFRDCWDYLTKYSLPYHPLHDQGFPSIGDVQSTIPVPREKWFEYAGER  300
EcPAPR   RANLPVLAIQ----------RGVFKVLPIIDWDNRTIYQYLQKHGLKYHPLWDEGYLSVGDTHTTRKWEPG-----MAEE  228
         *                       *  *            *         *   *  * *    *             *

AtAPR2   EGRWWWED----AKAKECGLHKGNIK--EEDGAADSKPAAVQEIFESNNVVALSKGGVENLLKLENRKEAWLVVLYAPWC  373
PpAPR    EGRWWWED----AKAKECGLHKGNLDGVAESSNSLGEKTEVADLFISENVVTFGREDMEALVKGENRDKSSLVVLYAPWC  385
PpPAPR   SGRFQGLTNPDGSAKTECGIHVGGRT                                                        326
EcPAPR   ETRFFG-------LKRECGLHEG                                                           244
           *             *** * *

AtAPR2   PFCQAMEASYIELAEKLAGKGVKVAKFRADGEQKEFAKQELQLGSFPTILLFPKRAPRAIKYPSEHRDVDSLMSFVNLLR  453
PpAPR    SFSQAMDESYNDVAEKLAGSNVVVGKFNADGAQKAYAKENLQLQSYPTVLFLPKNSSQIIKYPSENREVDALLGFVQALQ  465
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FIG. 7. **Comparison of amino acid sequences of APS and PAPS reductases.** The sequences of APR2 from *A. thaliana* (*AtAPR2*), APR (*PpAPR*) and PAPS reductase (*PpPAPR*) from *P. patens*, and PAPS reductase from *E. coli* (*EcPAPR*) were aligned with the program CLUSTAL. *Asterisks* identify identical residues, and *arrows* mark the additional Cys in APS reductases. The conserved APR and PAPS reductase signature is *underlined*.

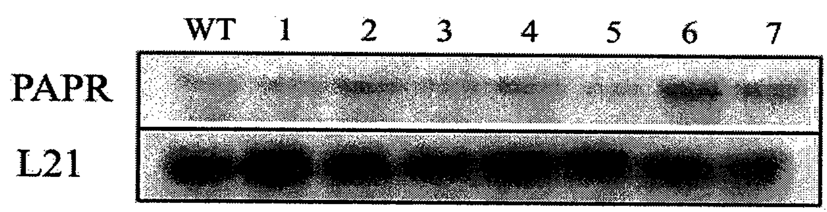

FIG. 8. **Expression of PAPS reductase.** Total RNA was extracted from protonema tissue of wild type *P. patens* (*WT*) and 7 Δapr knockouts: 11-3-3 (*lane 1*), 11-3-11 (*lane 2*), 11-3-12 (*lane 3*), 12-4-2 (*lane 4*), 12-4-3 (*lane 5*), 12-4-29 (*lane 6*), and 12-7-7 (*lane 7*). 5 μg of RNA were separated on 1% agarose in the presence of formaldehyde, blotted onto Hybond-N nylon membrane, and hybridized with ^{32}P-labeled cDNA fragments of PAPS reductase (PAPR) and L21.

Unfortunately, the final evidence that the EST indeed codes for PAPS reductase, *i.e.* the detection of PAPS reductase activity in moss extracts, is still missing. In *P. patens* extracts, both wild type and Δapr knockouts, sulfite production from PAPS was detectable neither with *E. coli* thioredoxin and glutaredoxin nor with recombinant thioredoxins m and f from spinach. Alternative cofactors might thus be required for the PAPS reduction, or the enzyme might be inhibited by dithioerythritol (used in the assay to keep the thioredoxin reduced) because *in vivo* thioredoxin is reduced by ferredoxin-thioredoxin reductase (23). The necessity of another unknown factor for the enzyme activity also cannot be excluded. The establishment of an assay for the PAPS reductase from *P. patens* will be a most important aim of further experiments.

P. patens is the first plant species wherein molecular evidence for PAPS reductase exists. No homologous genes are found in the *Arabidopsis* genome or in any plant EST data base, although there are many more EST sequences in the GenBank™ from several species than from *P. patens*. It is thus plausible to consider the PAPS reductase in *P. patens* to be an exception and not the rule for the organization of sulfate assimilation in plants. Both APR and PAPS reductase probably occurred in the last common ancestor of mosses and vascular plants. The latter was then probably lost in evolution and the diversification of higher plants, but both genes survived in *P. patens* and perhaps in other lower plants. Therefore, it would be very interesting to survey other mosses and liverworts for the presence of PAPS reductase to confirm this theory. Alternatively, the PAPS reductase gene could have been transferred to the *P. patens* genome by horizontal gene transfer as proposed for the evolution of APS and PAPS reductases in cyanobacteria (36). In any case, sulfate assimilation in *P. patens* differs significantly from that in higher plants, and the regulation of this pathway and above all the coordination of APS- and PAPS-dependent routes will certainly become a subject of new studies.

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